FEBS Letters 586 (2012) 2411–2416

journal homepage: www.FEBSLetters.org

The proline metabolism intermediate Δ^1-pyrroline-5-carboxylate directly inhibits the mitochondrial respiration in budding yeast

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ARTICLE INFO

Article history:
Received 16 April 2012
Revised 23 May 2012
Accepted 29 May 2012
Available online 12 June 2012

Edited by Judit Ovádi

Keywords:
Proline metabolism
Δ^1-Pyrroline-5-carboxylate
Oxidative stress
Mitochondria
Superoxide anion
Respiratory chain
Saccharomyces cerevisiae

ABSTRACT

The proline metabolism intermediate Δ^1-pyrroline-5-carboxylate (P5C) induces cell death in animals, plants and yeasts. To elucidate how P5C triggers cell death, we analyzed P5C metabolism, mitochondrial respiration and superoxide anion generation in the yeast *Saccharomyces cerevisiae*. Gene disruption analysis revealed that P5C-mediated cell death was not due to P5C metabolism. Interestingly, deficiency in mitochondrial respiration suppressed the sensitivity of yeast cells to P5C. In addition, we found that P5C inhibits the mitochondrial respiration and induces a burst of superoxide anions from the mitochondria. We propose that P5C regulates cell death via the inhibition of mitochondrial respiration.

1. Introduction

Accumulation of Δ^1-pyrroline-5-carboxylate (P5C), which is an intermediate in proline metabolism, induces cell death in plants and yeasts [1,2]. For animal cells, the dinitrophenylhydrazine-P5C compound exhibited the ability to inhibit cell growth, although free, acid extracted P5C was much less effective [3]. Genetic approaches, such as the overexpression of proline oxidase (proline → P5C) and a deficiency of P5C dehydrogenase (P5C → glutamate), suggested that P5C induces oxidative stress in cells. Overexpression of proline oxidase induced apoptosis through the generation of reactive oxygen species (ROS) in mammalian cells [4,5]. The growth of a mutant deficient in P5C dehydrogenase was inhibited by a high concentration of proline through the generation of ROS [2,6]. These effects were probably due to an increase in the intracellular P5C level [2]. P5C accumulation is suggested to induce the formation of ROS, which could directly or indirectly trigger oxidative stress by unknown mechanisms. In fact, direct treatment with P5C induces cell death and ROS generation in plants and yeasts [7,8]. There are two possible mechanisms for P5C that affects the cellular phenotype: (i) ROS is spontaneously generated from P5C or its equilibrium compound glutamate-γ-semialdehyde (GSA), which is a very unstable compound; (ii) ROS is produced through the enzymatic reactions in the P5C metabolic pathway; for example, proline oxidase (Put1) localized in the mitochondrial inner membrane requires oxygen molecules and cytochrome *c*, so it is possible to produce ROS through this reaction. However, the relationship between P5C accumulation and ROS generation is poorly understood.

The budding yeast *Saccharomyces cerevisiae* Σ1278b strain has the *MPR1* and *MPR2* genes (sigma 1278b gene for proline-analog resistance) encoding the *N*-acetyltransferase Mpr1 that acetylates P5C, or more likely, GSA, and the proline toxic analog, azetidine-2-carboxylate (AZC) [2,9,10]. Only one base change occurs between *MPR1* and *MPR2*, and both genes have similar functions. Gene disruption analysis revealed that Mpr1 mediates the L-proline and L-arginine metabolic pathways by acetylating P5C or GSA (Fig. 1), leading to protection against oxidative stress, such as exposure to high temperature [8]. In addition, we also found that *mpr1/2*-disruptant accumulated intracellular levels of P5C and was hypersensitive to direct treatment with P5C, suggesting that Mpr1 is involved in P5C detoxification [2,8]. Therefore, the yeast *mpr1/2*-disrupted strain would be a good model organism for elucidation of the P5C-derived mechanism of cell death.

In this study, we analyzed the action mechanism of P5C using *S. cerevisiae mpr1/2*-disrupted strain, which is hypersensitive to P5C.

Abbreviations: P5C, Δ^1-pyrroline-5-carboxylate; GSA, glutamate-γ-semialdehyde; ROS, reactive oxygen species; AZC, L-azetidine-2-carboxylate; TTC, 2,3,5-triphenyltetrazolium chloride; DCF-DA, 2′,7′-dichlorofluorescin diacetate

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0014-5793/$36.00
http://dx.doi.org/10.1016/j.febslet.2012.05.056

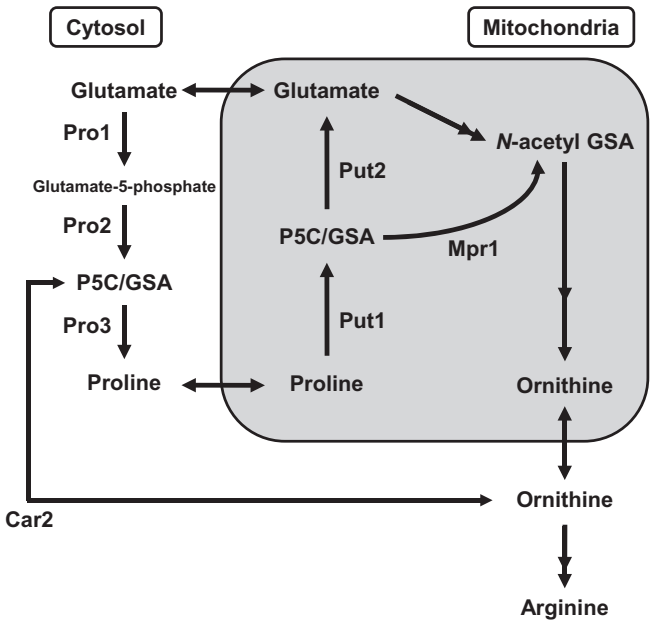

Fig. 1. Metabolic pathway of proline and arginine in *Saccharomyces cerevisiae*. Protein names: Pro1, γ-glutamyl kinase; Pro2, γ-glutamyl phosphate reductase; Pro3, P5C reductase; Put1, proline oxidase; Put2, P5C dehydrogenase; Mpr1; P5C/GSA *N*-acetyltransferase.

P5C-triggered cell death was shown to be independent of classical P5C metabolism. Interestingly, the sensitivity of yeast cells to P5C was cancelled by a defect in mitochondrial respiration. In addition, we found that P5C directly inhibits the mitochondrial respiration and induces a burst of superoxide anions from the mitochondria. Here, we propose that P5C is a direct inhibitor of mitochondrial respiration, leading to intracellular ROS generation in yeast.

2. Materials and methods

2.1. Strains, plasmids and culture media

The yeast *S. cerevisiae* strains with a Σ1278b background used in this study were L5685 (*MATa ura3-52 trp1 MPR1 MPR2*) [9], LD1014 (*MATa ura3-52 trp1 mpr1::URA3 mpr2::TRP1*) [9], LD1014ura3 (*MATa ura3-52 trp1 mpr1::URA3 mpr2::TRP1 ura3*) [2], LD1014*Δput1* (*MATa ura3-52 trp1 mpr1::URA3 mpr2::TRP1 put1::URA3*) [2], LD1014*Δput2* (*MATa ura3-52 trp1 mpr1::URA3 mpr2::TRP1 put2::URA3*) [2], LD1014*Δpro3* (*MATa ura3-52 trp1 mpr1::URA3 mpr2::TRP1 pro3::URA3*), LD1014*Δcar2* (*MATa ura3-52 trp1 mpr1::URA3 mpr2::TRP1 car2::URA3*) and LD1014ρ^0 (*MATa ura3-52 trp1 mpr1::URA3 mpr2::TRP1 rho^0*). Plasmid YEp24 (ATCC37051) was used for the construction of disruption cassette for *PRO3* and *CAR2*. The centromere-based low-copy-number plasmids pRS414 and pRS416 (Stratagene) harboring *TRP1* and *URA3*, respectively, were used for complementing the auxotrophic markers.

The media used for the growth of *S. cerevisiae* were a synthetic minimal medium SD (2% glucose and 0.67% Bacto yeast nitrogen base without amino acids (Difco Laboratories)) and a nutrient medium YPD (2% glucose, 1% yeast extract, and 2% peptone). When necessary, 2% agar was added to solidify the medium.

2.2. Construction of LD1014*Δpro3*, LD1014*Δcar2* and LD1014ρ^0

To construct LD1014*Δpro3* or LD1014*Δcar2*, the integration cassette was amplified by PCR with primers PRO3disURA3-Fw (ATG ACT TAC ACA TTG GCA ATT TTA GGC TGC GGT GTT ATG GGT TTG ACA GCT TAT CAT CGA) and PRO3disURA3-Rv (CTA TTT CTT CTT TTG GCC TAA TTG TGA CGC AAC ACG GGC TTA ACT GTG ATA AAC TAC CGC) or CAR2disURA3-Fw (CAC AAC ACC AAT GTC CGA AGC TAC CCT CTC CTC CAA GCA AGT TTG ACA GCT TAT CAT CGA) and CAR2disURA3-Rv (CGT ATT AAT ATT ATA ACA GAT CGA TAC ACT TGG CAA TGG TTA ACT GTG ATA AAC TAC CGC) using YEp24 as a template, respectively (the underlining indicates the sequences upstream of the initiation codon and downstream of the termination codon of the *PRO3* or *CAR2* gene, respectively). This PCR fragment was integrated into the *PRO3* and *CAR2* locus in LD1014ura3 by transformation. The Ura$^+$ phenotype was screened and the correct disruption event was verified by PCR using chromosomal DNA of each mutant as a template. For the *PRO3* disruption, proline was used as a sole nitrogen source.

To construct LD1014ρ^0, cells lacking mitochondrial DNA are isolated as follows [11]. LD1014 cells were cultured in SD plus 25 μg/ml ethidium bromide (filter-sterilized). After culturing to the stationary growth phase, culture was streaked for single colonies on YPD. The *rho^0* strain was selected by assimilation of glycerol, DAPI stain and 2,3,5-triphenyltetrazolium chloride (TTC) test.

2.3. Preparation and treatment of P5C/GSA

DL-P5C was freshly synthesized when needed by periodate oxidation of DL-hydroxylysine and purified using Dowex 50 (Dow Chemical), as described previously [12]. In our experiment, it was suggested that 1.25 mM P5C in 1.0 N HCl is stable for one month at 4 °C. Yeast cells were grown to the exponential phase (OD_{600} of 1.0) in SD medium at 25 °C and subjected to different concentrations of P5C for 2 h in SD medium. The effect of P5C on yeast was determined by cell viability and intracellular ROS level.

2.4. Cell viability assay

P5C-treated cells were diluted in distilled water, and aliquots were plated on YPD plates. After incubation at 30 °C for 2 days, the survival rates were expressed as percentages, calculated as follows: (no. of colonies after exposure to P5C treatment)/(no. of colonies with no treatment) × 100.

2.5. Measurement of intracellular ROS level

The ROS level induced in a cell during the P5C treatment was measured with the oxidant-sensitive probe 2′,7′-dichlorofluorescin diacetate (DCF-DA) (Molecular Probes). Exponential yeast cells were incubated at 25 °C for 30 min in SD medium containing 10 μM DCF-DA in the dark and then subjected to 10 μM P5C for 2 h. The harvested cells were washed twice with 50 mM potassium phosphate buffer (pH 7.4), resuspended in 500 μl of distilled water and disrupted with glass beads in a Multi-Beads Shocker (Yasui Kikai). Cell extracts (50 μl) were mixed in 450 μl of distilled water, and the fluorescence was measured with λ_{EX} = 504 nm and λ_{EM} = 524 nm using a fluorescence spectrophotometer (F-7000; Hitachi). The value of λ_{EM} = 524 nm was normalized by protein in the mixture. The protein concentration was determined by the Bradford assay. Intensity of fluorescence of strain L5685 with no treatment was relatively taken as 100%.

2.6. Measurement of mitochondrial ROS generation

Superoxide anions were detected with a water-soluble tetrazolium salt, WST-1 (Dojindo). WST-1 is reduced by superoxide anion to a stable water-soluble formazan, which exhibits absorbance at 450 nm. The mitochondria were isolated from L5685 as descried previously [13]. The purified mitochondria (0.3 mg/ml) were incubated with P5C (17 μM) in 20 mM HEPES–KOH buffer (pH 7.4)

containing 0.6 M mannitol, 800 μM WST-1, and 10 mM succinate (respiration substrate) at 30 °C for 30 min. The absorbance at 450 nm was determined with a DU-640 spectrophotometer (Beckman Coulter). The generated superoxide anions were calculated using the molar absorption coefficient ($\varepsilon = 1.1 \times 10^4\ M^{-1}\ cm^{-1}$).

2.7. Measurement of mitochondrial respiration activity

The mitochondrial respiration activity was determined by oxygen consumption using the Clark-type oxygen electrode (Digital Oxygen System Model-10, Rank Brothers). The mitochondria were isolated from L5685 as descried previously [13] using lactate as a sole carbon source. The purified mitochondria (0.3 mg/ml) were incubated with 20 mM HEPES–KOH buffer (pH 7.4) containing 0.6 M mannitol at 30 °C for 120 s and treated with 10 mM succinate (respiration substrate) to start respiration. After 180 s, various concentrations of P5C, 2 mM KCN (respiration inhibitor), 100 μM proline, 100 μM glutamate or 20 mM HEPES–KOH buffer (control) was added to examine the effect of P5C on mitochondrial respiration. All measurements were performed at 30 °C.

3. Results

3.1. P5C-mediated cell death is not due to P5C metabolism in yeast

We previously found that the *mpr1/2*-disrupted strain LD1014 is more sensitive to the direct treatment with P5C than the wild-type strain L5685 [8]. The accumulation of P5C is suggested to induce the formation of ROS, which could directly or indirectly trigger oxidative stress by unknown mechanisms. Therefore, we analyzed the P5C-derived mechanism of cell death using LD1014.

First, we examined the effect of P5C-metabolic enzymes on the sensitivity of yeast cells to P5C. Three proteins are involved in the metabolism of P5C in *S. cerevisiae*; Pro3 (P5C reductase; P5C → proline), Put2 (P5C dehydrogenase; P5C → glutamate) and Car2 (ornithine aminotransferase; P5C ↔ ornithine) (Fig. 1). In addition, we also focused on P5C-proline cycle catalyzed by proline oxidase (Put1 in *S. cerevisiae*) and P5C reductase. This cycle was suggested to maintain the redox balance in the cells, but to cause the production of ROS [14,15]. For the above purposes, we determined the cell viability and intracellular ROS level of L5685 (wild-type), LD1014 (*Δmpr1/2*), LD1014*Δput1* (*Δmpr1/2, Δput1*), LD1014*Δput2* (*Δmpr1/2 Δput2*), LD1014*Δpro3* (*Δmpr1/2 Δpro3*) and LD1014*Δcar2* (*Δmpr1/2 Δcar2*) after treatment with P5C (Fig. 2). In agreement with our previous data [8], the cell viability of LD1014 was significantly lower than that of L5685 after exposure to P5C, whereas little effect of P5C was observed in L5685 up to 100 μM (Fig. 2A). In correspondence with the data of survival rates, the intracellular ROS level of LD1014 was higher than that of L5685 (Fig. 2B). Interestingly, P5C induced cell death in all disruptant strains in proportion to P5C concentration, the level being similar to those in LD1014. However, LD1014*Δput2* and LD1014*Δpro3* showed higher sensitivity to P5C than LD1014, probably due to an increase in P5C content caused by the deficiency of Put2 or Pro3, in addition to the lack of Mpr1/2. These results suggest that classical P5C metabolism is independent of P5C-mediated cell death in yeast.

3.2. Mitochondrial respiration is involved in P5C-mediated cell death in yeast

The mitochondrial respiratory chain has been shown to be a main source of ROS generation in cells [16,17]. Therefore, we constructed the yeast respiratory-defective strain, LD1014*rho*^{0}, and checked the cell viability and intracellular oxidation level in the presence of P5C (Fig. 2). It should be noted that LD1014*rho*^{0} cells were tolerant to P5C up to 100 μM in terms of the cell viability (Fig. 2A) and ROS level (Fig. 2B). This result indicates that mitochondrial respiration is involved in the sensitivity of yeast cells to P5C.

Next, we purified mitochondria from L5685 cells and examined the effect of P5C on the mitochondrial ROS generation (Fig. 3). Even in the absence of P5C, a small amount of superoxide anion was detected in the mitochondria. After incubation with P5C, the mitochondria exhibited an approximately twofold increase in superoxide anion, as compared to mitochondria in the absence of P5C. Interestingly, we found that superoxide anion generation did not occur when succinate, which is a respiratory substrate, was omitted from the reaction mixture. It is also intriguing to see whether superoxide anions are spontaneously produced or generated through the reaction with succinate. Removal of the mitochondria from the reaction mixture canceled the accumulation of superoxide anions in the mitochondria in the presence or absence of P5C. These results strongly suggest that P5C inhibits the function of the respiration chain in the mitochondria, leading to intracellular accumulation of ROS. We also performed the same experiments with mitochondria from LD1014 cells, expecting a higher production of superoxide anion, but no clear increase in the production of this anion was observed over that in the mitochondria from L5685 cells. This finding suggests that under the conditions of these in vitro experiments the limited availability of acetyl CoA renders P5C/GSA acetylation little operative.

3.3. P5C inhibits mitochondrial respiration activity in a dose-dependent manner in yeast

It is known that inhibition of the respiration chain induces ROS formation in the mitochondria [16,17]. Considering that P5C has an inhibitory effect on the mitochondrial respiratory activity, we measured the amount of oxygen consumption and the rate of this consumption in the mitochondria using an oxygen electrode (Fig. 4). When the respiratory chain was activated by the addition of succinate, we found that respiratory activity was inhibited by P5C in a dose-dependent manner (Fig. 4A). However, proline and glutamate, which are structurally similar to P5C, had no effect on the oxygen consumption (Fig. 4A). The oxygen consumption rate significantly decreased in the presence of P5C (Fig. 4B). The concentration of P5C needed to achieve 50% inhibition of oxygen consumption (IC_{50}) was 23.8 μM, indicating that P5C inhibits the mitochondrial respiration activity, leading to ROS generation.

4. Discussion

Recently, the proline cycle or P5C-proline cycle, which is the cytosolic reduction of P5C generated by mitochondrial proline oxidation, was proposed in mammalian and plant cells [14,15]. This cycle regulates the intracellular P5C level that is not immediately oxidized to glutamate by the mitochondrial P5C dehydrogenase and exported to the cytosol. Therefore, this cycle is essential for cell survival during various stresses in terms of preventing P5C accumulation. The further reduction of P5C to proline depends on the cytosolic P5C reductase activity and the possible existence of unidentified P5C transporter(s) in the mitochondrial membrane. However, hyperactivity of the cycle, such as the addition of excess proline, results in superoxide anion formation in the mitochondria by delivering electrons to oxygen. Therefore, normal oxidation of P5C to glutamate by P5C dehydrogenase is the key to preventing P5C-proline intensive cycling and avoiding ROS generation [14]. As shown in Fig. 2, LD1014*Δput1* was also sensitive to P5C/GSA, suggesting that P5C/GSA is unlikely to affect the proline oxidase

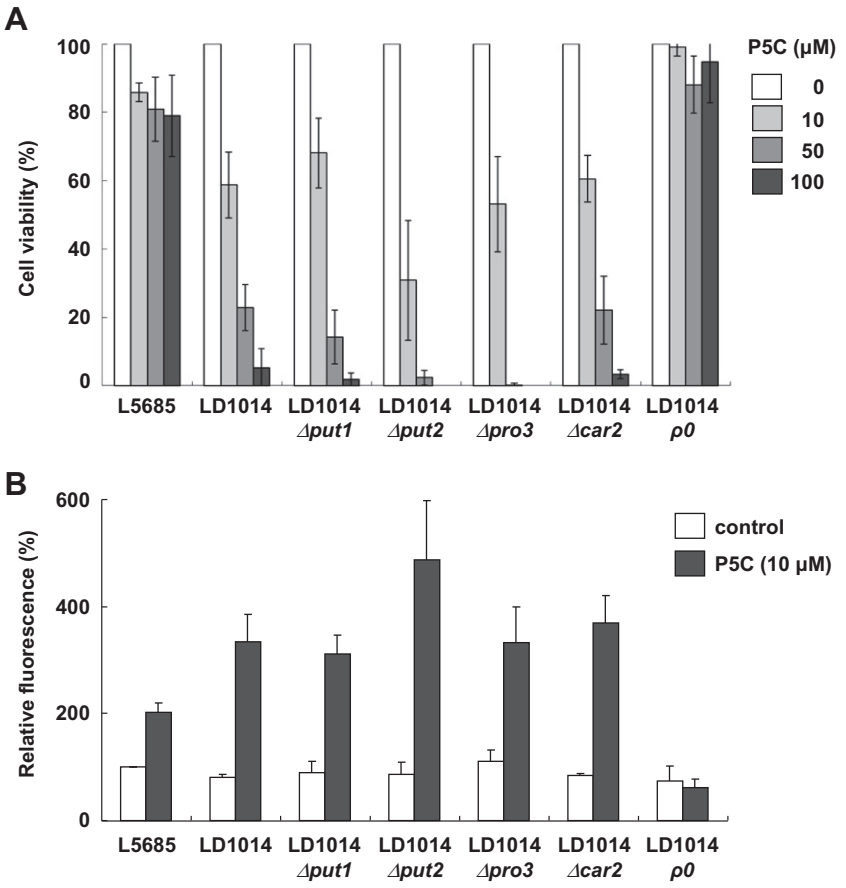

Fig. 2. Effect of P5C on the cellular phenotype. (A) Cell viability of strains L5685 (pRS416, pRS414), LD1014, LD1014*Δput1*, LD1014*Δput2*, LD1014*Δpro3*, LD1014*Δcar2* and LD1014ρ^0. Cells were grown in SD at 25 °C to OD_{600} of 1.0, and incubated with different concentrations (0–100 μM) of P5C at 25 °C for 2 h. Cell viability was measured by counting colony-forming cells on YPD plate. The values are the means and standard deviations of nine independent experiments. (B) Intracellular oxidation level of L5685 (pRS416, pRS414), LD1014, LD1014*Δput1*, LD1014*Δput2*, LD1014*Δpro3*, LD1014*Δcar2* and LD1014ρ^0. Cells were grown in SD at 25 °C to OD_{600} of 1.0, and incubated with P5C. Intracellular ROS level was detected by DCF-DA. The intensity of fluorescence of each strain was measured after incubation with 10 μM P5C at 25 °C for 2 h. Intensity of fluorescence of L5685 in the absence of P5C was relatively taken as 100%. The values are the means and standard deviations of nine independent experiments.

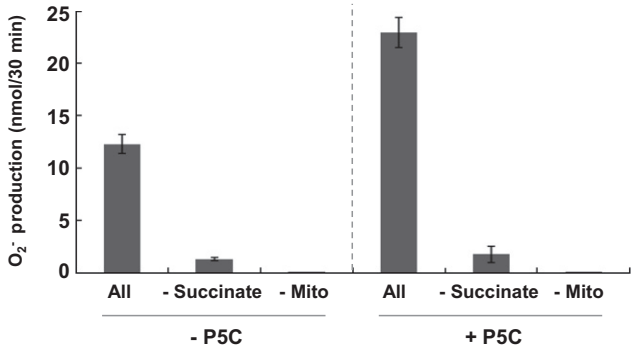

Fig. 3. P5C-mediated ROS production. Superoxide anion production (nmol/30 min) from the mitochondria was measured by the reduction of WST-1. Succinate as a respiration substrate (-Succinate) or purified mitochondria (-Mito) was omitted from the reaction mixture (All) in the presence or absence of 17 μM P5C. The values are the means and standard deviations of three independent experiments.

(Put1) activity. In addition, we do not think that proline-derived electrons are used to reduce oxygen, because the *rho*^{0} strain cannot utilize proline as the sole nitrogen source (data not shown). If these electrons from proline are transferred to oxygen, proline utilization may occur in the *rho*^{0} cells.

It is unknown whether such a system that reduces the intracellular P5C level is conserved in *S. cerevisiae*. However, we previously suggested that P5C accumulation induces cell death in yeast under oxidative stress conditions such as freezing and heat-shock treatment due to the formation of ROS [2,18] and that Mpr1 regulates the ROS level under P5C-induced oxidative stress conditions [2]. Here, we analyzed the P5C-derived mechanism of ROS generation in yeast. According to our recent study, it is estimated that the P5C concentration in yeast cells is about 200 μM under the non-stressed condition, but is elevated to about 300 μM after temperature upshift [8]. This increase in P5C level (100 μM) caused by high temperature stress is suggested to be adequate to induce cell death in LD1014 lacking Mpr1/2. In this study, we did not measure the level of intracellular P5C, but the proline transporter(s) on the plasma membrane, such as Gap1, Put4, Agp1 and Gnp1, may transport P5C into yeast cells. Inside the cell, Put2 and/or Pro3 may detoxify P5C/GSA by converting it into glutamate and/or proline, respectively. Many yeasts and fungi contain the homologous genes of *MPR1*, suggesting that *MPR1* is widely present in eukaryotic microorganisms [19,20]. We previously found that Mpr1 protects yeast cells by regulating ROS levels under oxidative stress conditions, such as H_2O_2, freezing, ethanol or high-temperature treatment [2,8,19–22]. Recently, we revealed that Mpr1 converts P5C/GSA into *N*-acetyl-GSA for arginine synthesis in the mitochondria, indicating that Mpr1 mediates the proline and arginine metabolic pathways [8]. More importantly, Mpr1-dependent arginine synthesis confers stress tolerance. In addition, we propose here that Mpr1 is a novel antioxidant enzyme that acetylates toxic P5C involved in ROS generation.

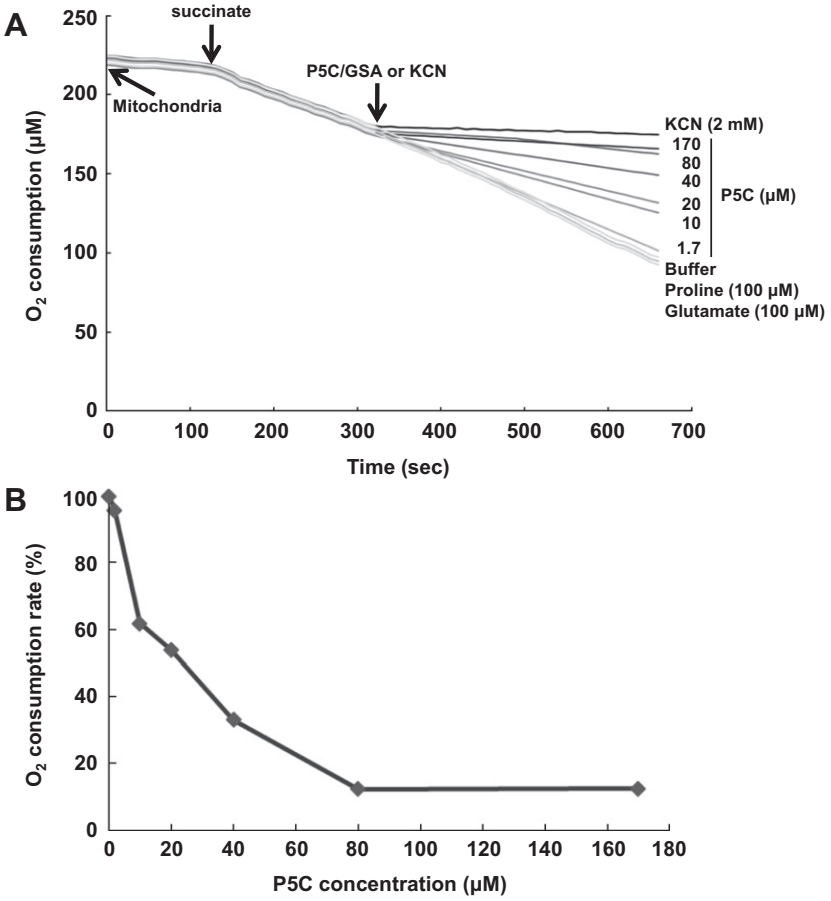

Fig. 4. Inhibition of the mitochondrial respiration by P5C. (A) Inhibition of oxygen consumption by P5C. The mitochondrial oxygen consumption (μM) was determined with an oxygen electrode. The data shown are from one experiment, but are representative of three independent experiments. (B) Relative oxygen consumption under various concentrations of P5C. Oxygen consumption (% of P5C-untreated mitochondria) was calculated from rates after the addition of P5C in Fig. 3A. The oxygen consumption in P5C-untreated mitochondria was 0.14 μmol/min/mg. The data shown are from one experiment, but are representative of three independent experiments.

We showed that P5C-mediated cell death is not due to P5C/GSA-metabolism, but P5C directly inhibits the mitochondrial respiration that is a major source of ROS. Within the respiratory chain, complex I (NADH dehydrogenase site) and complex III (the Qi site) are considered to be the principal sites for ROS generation based on experiments using mammalian mitochondria with respiratory inhibitors, such as KCN, Rotenone and Antimycin A [16,17,23]. Here we showed the occurrence of superoxide anion generation by intact mitochondria with a complex II substrate, succinate. This result suggests that P5C inhibits the respiratory chain at complex II (succinate dehydrogenase) or downstream of this complex (complexes III and/or IV (cytochrome *c* oxidase)). We will identify the targeted complex for P5C in the respiratory chain through biochemical approaches. In Fig. 4A, although proline has no apparent effect on O_2 consumption, what would happen with P5C/GSA if proline can be used as the substrate rather than succinate? Probably due to the competitive inhibition with proline and P5C/GSA, it must be quite difficult to interpret the result. Proline is not a good respiratory substrate in yeast cells, so we may obtain a similar result that superoxide anion generation does not occur when succinate is omitted from the reaction mixture.

The IC_{50} value for P5C, for its reduction of oxygen consumption, was 23.8 μM, which is lower than the intracellular level during normal metabolism (approximately 200 μM), suggesting that mitochondrial respiration can be stopped efficiently by increase in P5C content. Since the in vivo P5C concentration is approximately eightfold higher than its half-inhibitory concentration for mitochondrial respiration, it might be expected that the cells were unable to respire. However, we do not consider this possibility because the mitochondrial concentration of P5C in vivo may be much lower than that in the cytosol, because of the operation in the mitochondria of Put2 and Mpr1. In contrast, in our in vitro experiments with isolated mitochondria these enzymes may be little operative due to limited availability of NADPH and acetyl CoA.

Mitochondrial P5C may be involved in cellular signaling. This result substantiates the proposed hypothesis that P5C is a primary inducer of p53-mediated apoptosis and ROS-dependent autophagy proposed in mammals [15,24]. Recently, the cytotoxicity of formaldehyde and acetoaldehyde containing the aldehyde group has been attributed to their ability to impair mitochondrial function, thus generating ROS [25,26]. Therefore, it may be reasonable that P5C or, more likely, its equilibrium compound GSA with an unstable aldehyde group, attacks the mitochondrial respiratory chain. P5C is suggested to regulate cell death via the inhibition of the respiratory chain. The mechanism of P5C-mediated cell death is currently unknown. Judging from the observation that superoxide anions are generated from the mitochondria, it is intriguing to see whether P5C induces or activates apoptosis in yeast cells. Thus, a yeast *mpr1/2* disrupted strain (LD1014) that is hypersensitive to P5C may be a promising model organism for the elucidation of the P5C-derived mechanism of cell death.

Acknowledgments

We thank N. Yoshida of our laboratory for the discussion on this work and G. Fink (Massachusetts Institute of Technology,

Cambridge, MA) for providing strain L5685. This work was supported by a Grant-in-Aid for Scientific Research (B) (22380061) from Japan Society for the Promotion of Science to H.T. and by a Grant-in-Aid for Scientific Research on Innovative Areas from the Ministry of Education, Sciences, Sports and Technology (MEXT) to H.T.

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